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Journal of Chromatography A, 1009 (2003) 179–188
JOURNAL OF CHROMATOGRAPHY A
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Purification and partial characterization by matrix-assisted laser desorption ionization time-of-flight mass spectrometry of the recombinant transposase, TniA

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Abstract

A recombinant transposase, TniA, a basic DNA binding protein, was chromatographically purified and characterized by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) methods. *Escherichia coli* cells, overexpressing native TniA, were ultrasonically disrupted and the clarified supernatant was used as starting material for anion-exchange chromatography on SOURCE[1] 15Q 4.6/100 PE (Tricorn), at pH 7.5. This initial step was proven to be a fast and simple way of removing acidic proteins like proteases. TniA was collected from the flow-through fraction and applied onto HiTrap heparin HP 5 ml in order to capture the basic TniA. This was followed by cation-exchange chromatography through Mono S 5/50 GL (Tricorn), at pH 6.5 which resulted in a purity of TniA of about 95%. The molecular mass of TniA was determined to 62 869 rel. mol. mass units with MALDI-TOF-MS and the identity of the protein was confirmed by peptide mass fingerprinting of trypsin-digested TniA. Partial amino acid sequencing was achieved after derivatization of tryptic peptides using Ettan™ CAF™ MALDI Sequencing Kit and post source decay. The fact that transposases are DNA-binding and that many of them possess basic isoelectric point values suggest that the outlined purification protocol may serve as a general method for the purification of recombinant nontagged transposases and other basic DNA-binding proteins.

Keywords: Enzymes; Transposase; TniA

1. Introduction

Transposons are genetic elements that contribute to both short-term development and to long term evolution by their property of being movable between or within DNA molecules of both eukaryotes and prokaryotes. In prokaryotes these genetic elements often contain antibiotic resistance genes. The majority of transposons encode a cognate metal-binding enzyme—transposase—responsible for both the cleavage of the donor DNA and the subsequent joining of the transposon to the target [1]. The transposon Tn*5090*/Tn*402*, which carries the TniA gene, belongs to the Mu-like subfamily of transposons [2]. These elements use a two-step trans-

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[1]Please note that ÄKTA, SOURCE, HiTrap, HiPrep, Mono S, Ettan and CAF are trademarks of Amersham Biosciences Limited.

0021-9673/03/$ – see front matter
doi:10.1016/S0021-9673(03)00922-1

esterification mechanism that contains fewer steps than for some other transposons such as Tn*10* [1,3]. The Mu-like transposons activate their transposases by assembling it into a tetramer [1]. The transposase of Tn*5090*/Tn*402*, TniA, is assembled through contacts with several 19 base pair (bp) repeats on either of the transposon ends [4]. Apart from TniA, Tn*5090*/Tn*402* also encodes for two other transpositional proteins (TniB and TniQ) and a resolvase (TniC) [2,5]. The transposon displays a strongly biased target selectivity determined by an external target factor [6]. Because Tn*5090*/Tn*402* carries an integron (type 1), it has captured three gene cassettes, two of which mediate resistance to antimicrobial agents [1,7,8].

In this study, the transposase of Tn*5090*/Tn*402* was purified from a bacterial culture overproducing the protein [4]. The purification was carried out by the use of three chromatographic steps; non-capture anion exchange, affinity chromatography, and high-resolution cation exchange. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) were applied for the partial characterization of the purified transposase.

2. Experimental

2.1. Materials

Ile^{7}-Angiotensin III (Ang III), human adrenocorticotropic hormone fragment 18–39 (hACTH 18–39), bovine serum albumin (BSA), α-cyano-4-hydroxycinnamic acid, sinapinnic acid and NAP-5 columns were from Amersham Biosciences (Uppsala, Sweden). Sequencing grade trypsin was purchased from Promega (Madison, WI, USA). Acetonitrile (HPLC grade), trifluoroacetic acid (TFA) and phenylmethanesulfonyl fluoride (PMSF) were from Sigma (St. Louis, MO, USA). Tris(hydroxymethyl)

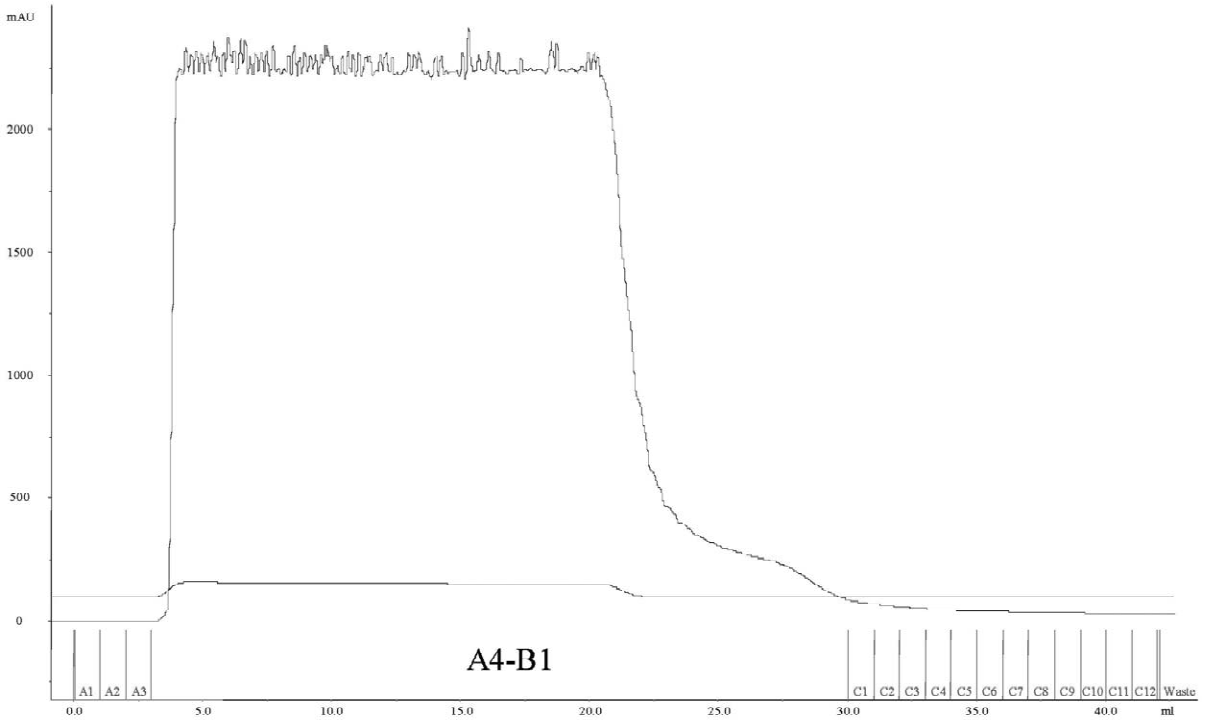


Fig. 1. Non-capture anion-exchange chromatography on Source 15 Q 4.6/100 PE (Tricorn). The flow-through fractions A4–B1 (27 ml) were collected.

aminomethane (Tris), EDTA, dithiothreitol (DTT), urea, 2-(*N*-morpholino)ethanesulfonic acid (MES), $MgCl_2$, NaCl and iodoacetamide were obtained from Merck (Darmstadt, Germany). ZipTip C_{18} was purchased from Millipore (Bedford, MA, USA).

2.2. Methods

2.2.1. Disruption of bacterial cells

Approximately 2 g of bacterial cells overexpressing the transposase was suspended in 20 ml lysis buffer (20 m*M* Tris–HCl, pH 7.5, 1 m*M* PMSF, 1 m*M* EDTA, 2 m*M* $MgCl_2$, and 1 m*M* DTT) and disintegrated by sonication in a Sonics vibra cell for 2 min. The crude extract was centrifuged (Beckman J2-21, rotor: JA-20, 10 000 rpm, 4 °C, 30 min) and the clarified supernatant was collected.

2.2.2. Chromatography and media

An ÄKTA Explorer 100 system controlled by UNICORN software (Amersham Biosciences) was used for protein purification and peak evaluation according to the manufacturer's instructions. Fractions were collected in a Frac 950 (Amersham Biosciences) equipped with a tube rack for 3 ml tubes. A 50-ml Superloop (Amersham Biosciences) was used for sample application. The chromatographic purification was carried out at 4 °C.

A 17.5-ml volume of the clarified supernatant was applied to a Source 15Q 4.6/100 PE (Tricorn) anion-exchange column at a flow-rate of 1 ml/min. The buffer used was 20 m*M* Tris–HCl, pH 7.5, 1 m*M* EDTA, 2 m*M* $MgCl_2$, 1 m*M* DTT. The basic TniA (theoretical isoelectric point, p*I* 9.34) was obtained in the flow-through fraction, which was collected. To capture the DNA-binding TniA, 28 ml of the flow-through from the first step was applied to HiTrap heparin HP 5 ml. Prior to this, the salt concentration of the sample was adjusted to approximately 0.15 *M* NaCl. The binding buffer used in the capture step was 20 m*M* Tris–HCl, pH 7.5, 1 m*M* EDTA, 2 m*M* $MgCl_2$, 1 m*M* DTT, 0.15 *M* NaCl. The elution buffer was the same as the binding buffer, except the NaCl concentration, which was 1 *M*. Bound protein

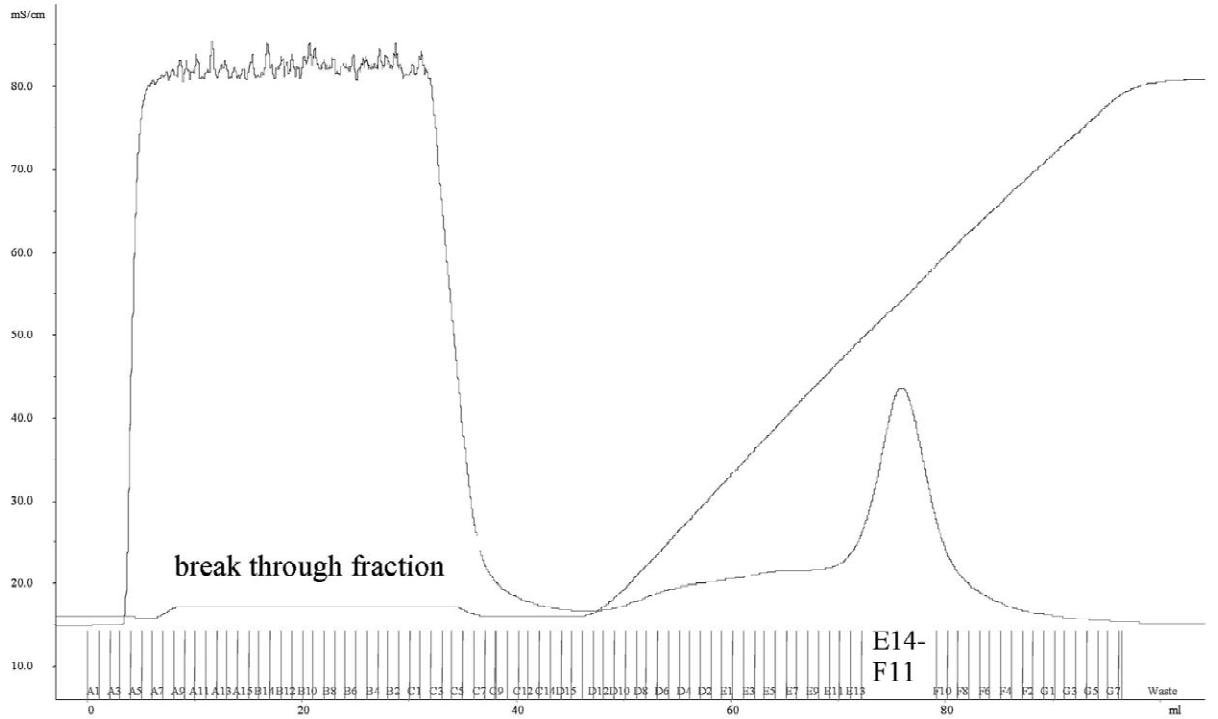


Fig. 2. Affinity chromatography on HiTrap heparin HP 5 ml. Fractions E14–F11 (7 ml) were pooled.

was eluted with a linear gradient of 10 column volumes (CVs) from 0 to 100% elution buffer, at a flow-rate of 2.5 ml/min. Fractions containing TniA were pooled and the buffer of the sample was exchanged to 20 m*M* MES, pH 6.5, 1 m*M* EDTA, 2 m*M* $MgCl_2$, 1 m*M* DTT using HiPrep 26/10 Desalting. After buffer exchange 14.5 ml of the sample was applied to the cation-exchange column Mono S 5/50 GL (Tricorn) as a high-resolution purification step, at a flow-rate of 1 ml/min. 20 m*M* MES, pH 6.5, 1 m*M* EDTA, 2 m*M* $MgCl_2$, 1 m*M* DTT was used as binding buffer. The elution buffer was binding buffer with an additional 1 *M* NaCl. Bound protein was eluted with a linear gradient of 20 CVs from 0 to 100% elution buffer.

2.2.3. SDS–PAGE

Fractions from all chromatographic steps were analyzed by SDS–PAGE under denaturing conditions in the PhastSystem and PhastGel Homogeneous 12.5 according to the recommendations from the manufacturer (Amersham Biosciences). Protein bands were visualized by staining with the PhastGel Protein Silver Staining Kit or PhastGel Blue R-350 also according to the manufacturer's recommended procedures.

2.2.4. MALDI-TOF-MS

All MS analyses were conducted with an Ettan MALDI-ToF Pro system (Amersham Biosciences) equipped with a quadratic field reflectron and a timed ion gate.

Analysis of intact proteins was performed in the linear mode with positive ionization at an acceleration voltage of 20 kV. Prior to MS analysis the transposase was desalted against ultrapure water using a NAP-5 column. The samples for MS analysis were prepared by the ''dried-droplet'' method. A 1-μl volume of the analyte was mixed with an equal volume of 0.9% TFA and 50% acetonitrile saturated with sinapinnic acid. A 0.3-μl aliquot of the mixture was dispensed onto the stainless steel MALDI sample slide for analysis.

Protein identification using peptide mass fingerprinting of trypsin-digested TniA was conducted in the reflectron mode with positive ionization at 20 kV.

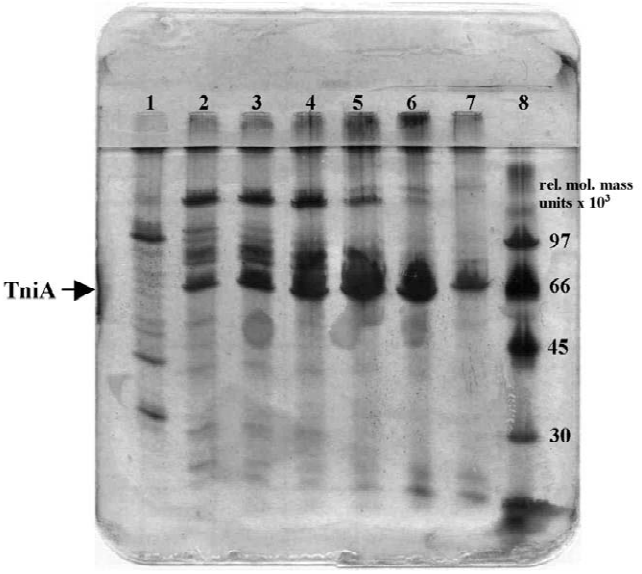


Fig. 3. Silver stained SDS–PAGE performed with PhastSystem on PhastGel Homogeneous 12.5 of fractions from the affinity chromatography step. Bands were visualized by silver staining. Lanes: 1=fraction E6; 2=fraction E13; 3=fraction E14; 4=fraction E15; 5=fraction F14; 6=fraction F 12; 7=fraction F9; 8=low-molecular-mass SDS marker kit (97, 66, 45, 30, 20.1 and $14.4 \cdot 10^3$ rel. mol. mass units).

The sample was in this case mixed with an equal volume of 50% acetonitrile, 0.5% TFA saturated with α-cyano-4-hydroxycinnamic acid and 0.3 μl was applied to the sample slide. Ang III and hACTH 18–39 were used for internal calibration.

Post source decay (PSD) was performed by first acquiring a reflectron mode spectrum which was internally calibrated. Based on this analysis, peaks were chosen for timed ion gating and the instrument was switched to PSD mode at an acceleration voltage of 20 kV. Due to the quadratic field reflectron of the instrument used, PSD spectra were obtained over the entire m/z range without the need for data stitching.

2.2.5. CAF MALDI sequencing

Derivatization of tryptic peptides of TniA was performed on ZipTip C_{18} according to the instructions in the Ettan™ CAF™ MALDI Sequencing Kit (Amersham Bioscences). The kit is based on chemistry developed by Keough et al. [9]. The tryptic peptides of TniA were first guanidinated resulting in the conversion of lysines to homoarginines and an increased peptide mass of 42 rel. mol. mass units [10]. This was followed by labelling with CAF reagent which adds a sulfonic acid group to the N-terminal of the peptides and thereby increasing their molecular mass by 136 rel. mol. mass units. After ionization by MALDI, the sulfonated N-terminus will be negatively charged through deprotonation which is balanced by the positive charge on the basic C-terminus (Arg). One additional proton is formed which randomly can move along the backbone of the CAF derivatized peptide resulting in an enhanced fragmentation towards b- and y-fragments during PSD [9]. Due to the negative charge at the N-terminus, the b-fragments will become neutral, resulting in a PSD spectrum consisting exclusively of y-ions thus simplifying the interpretation of acquired spectra. The amino acid sequence of a CAF derivatized peptide can thus be calculated manually from

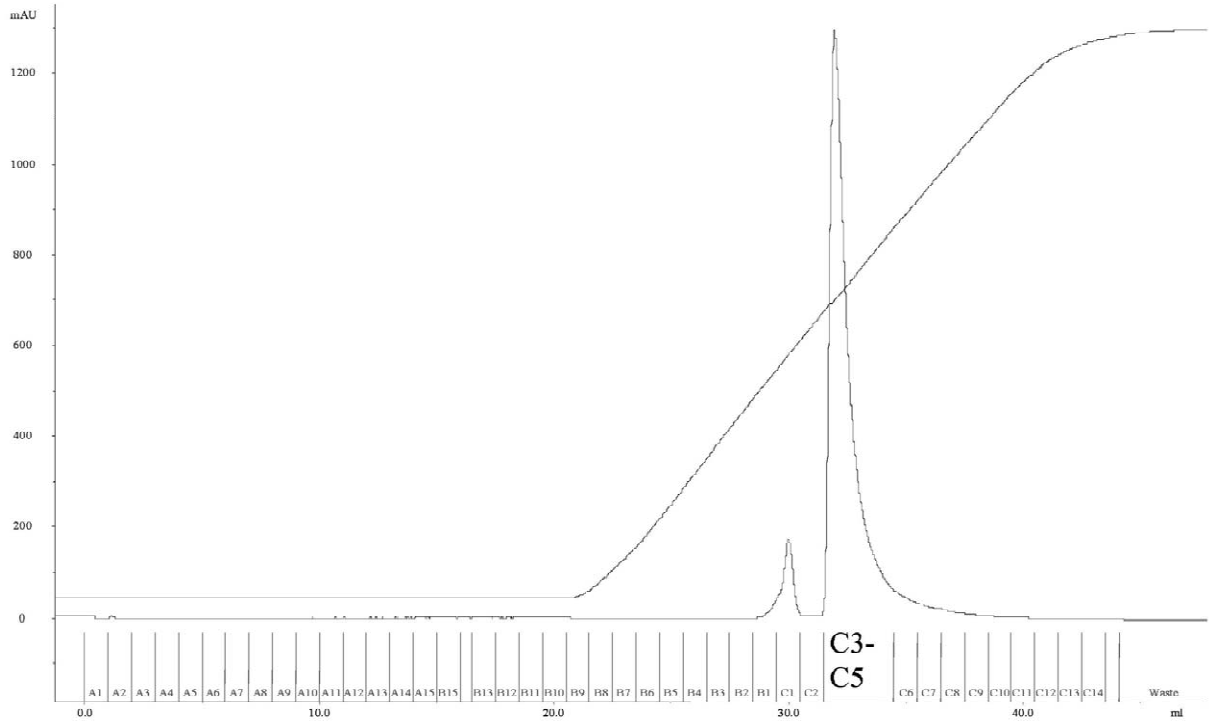


Fig. 4. Cation-exchange chromatography on Mono S 5/50 GL (Tricorn) of the desalted fractions from the affinity step. The fractions containing the major peak, C3–C5 (3 ml), were pooled and contained approximately 5.6 mg of TniA. The content of the first (small) peak is unknown, but SDS–PAGE demonstrated that it contained material with higher molecular mass than TniA (data not shown).

the mass differences between adjacent y-fragments. The derivatization step in the Ettan™ CAF™ MALDI Sequencing Kit is advantageous to use compared to the original chemistry reported by Keough and co-workers since the reactions can be performed in an aqueous environment [11–13].

3. Results and discussion

3.1. Chromatographic purification of TniA

As a first chromatographic step in the purification of TniA an anion-exchange column (Source 15Q 4.6/100) was used as a non-capture procedure as a highly efficient method to remove large amounts of acidic *Escherichia coli* proteins, e.g., protein degrading enzymes. This fast and simple method makes it possible to provide an environment in which the basic TniA is stable and minimizes the risk of proteolytic cleavage. This was found to be an effective method to protect the target protein from proteolytic degradation. Other purification protocols tried during the development of the method employed often resulted in lower yields due to degradation of the TniA (data not shown). TniA was obtained in the flow-through of the anion-exchange purification step (Fig. 1) and in a buffer system suitable for the next purification step, where the group-specific medium Heparin Sepharose HP [14] was used for capturing of the basic TniA. The transposase was eluted as a distinct and broad peak at ~0.5 *M* NaCl (Fig. 2). No transposase was found in the break-through fractions and the estimated purity of TniA was 85% as judged by the silver stained SDS–PAGE gel shown in Fig. 3. The pooled fractions containing TniA from the affinity chromatography step were desalted and a final purification step was performed by cation-exchange chromatography (Mono S 5/50 GL). From the resulting chromatogram in Fig. 4, two highly resolved peaks are visible. When the material in these peaks were examined by SDS–PAGE, the smaller peak was found to contain material with higher molecular mass than TniA (data not shown) while the major peak consisted almost exclusively of TniA (Fig. 5). A similar methodology was used by Bhikhabhai et al. for purification of recombinant reverse transcriptase [15].

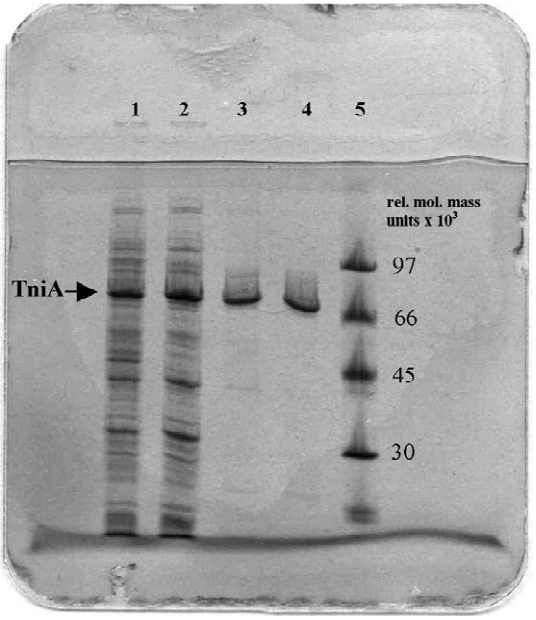


Fig. 5. SDS–PAGE performed with PhastSystem on PhastGel Homogeneous 12.5 of pooled fractions from different chromatographic purification steps of TniA. The gel was stained with Coomassie Blue. Lanes: 1=clarified extract; 2=flow-through fractions from the anion-exchange chromatography step; 3= fractions from the heparin affinity chromatography step; 4= fractions from the cation-exchange chromatography step; 5=low-molecular-mass SDS marker kit (97, 66, 45, 30, 20.1 and 14.4·10^3 rel. mol. mass units).

3.2. Analysis of the intact TniA by MALDI-TOF-MS

The molecular mass of the chromatographically purified TniA was determined by MALDI-TOF-MS operating in the linear mode with positive ionization after external calibration with BSA. The resulting spectrum is shown in Fig. 6. Three distinct peaks representing single, double and triple charged monomeric TniA with m/z (mass vs. charge) values of 62 480, 31 448, and 21 080, respectively, can be seen. The molecular mass calculated from these values results in 62 866 rel. mol. mass units which is in good agreement with 62 950 rel. mol. mass units which is the molecular mass of TniA calculated from

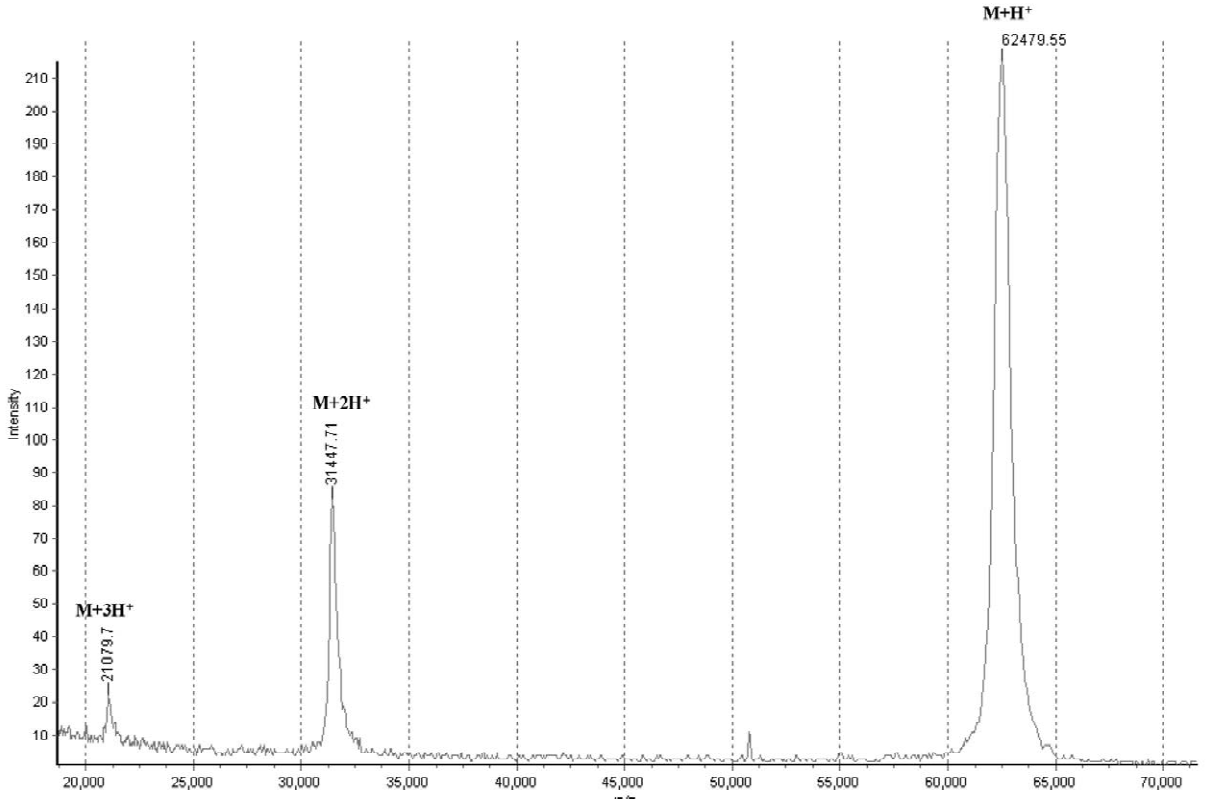


Fig. 6. MALDI-TOF-MS spectrum acquired in the linear mode of the intact TniA from the cation-exchange chromatography step. Approximately 1 pmol of the transposase was loaded on the MALDI sample slide. The peak at an m/z value of 62 480 represents the singly charged transposase. The peaks at m/z values of 31 448 and 21 080 represent double and triple charged transposase, respectively.

nucleic acid sequencing data [4]. The deviation of 0.1% from the predicted molecular mass is quite low and might lie within the statistical error of the method employed.

3.3. Identification of trypsin-digested TniA by MALDI-TOF-MS

In order to identify the chromatographically purified TniA, the transposase was digested with trypsin and the peptides so generated were analysed by MALDI-TOF-MS in the reflectron mode. The acquired m/z values were analyzed by Ettan MALDI-ToF Pro software which resulted in more than 40% coverage of the protein and assignment of TniA as the highest ranked candidate (Fig. 7). Taken together, the results from determinations of the intact TniA and peptide mass fingerprinting by MALDI-TOF-MS clearly demonstrate that the recombinant form of TniA is identical to its natural counterpart.

3.4. Determination of the sequences of two tryptic peptides of TniA by CAF-mediated MALDI-PSD

The amino acid sequences of two peptides generated from TniA after trypsin digestion were determined by MALDI-TOF-MS in the PSD mode after chemical derivatization using the Ettan™ CAF™ MALDI Sequencing Kit. As described in the Methods section, the tryptic digest of TniA was derivatized by guanidination and sulfonation. The peptides chosen for sequencing are denoted by arrows in Fig. 7 with underivatized m/z values of 801.4 (peptide 1) and 979.5 (peptide 2). After

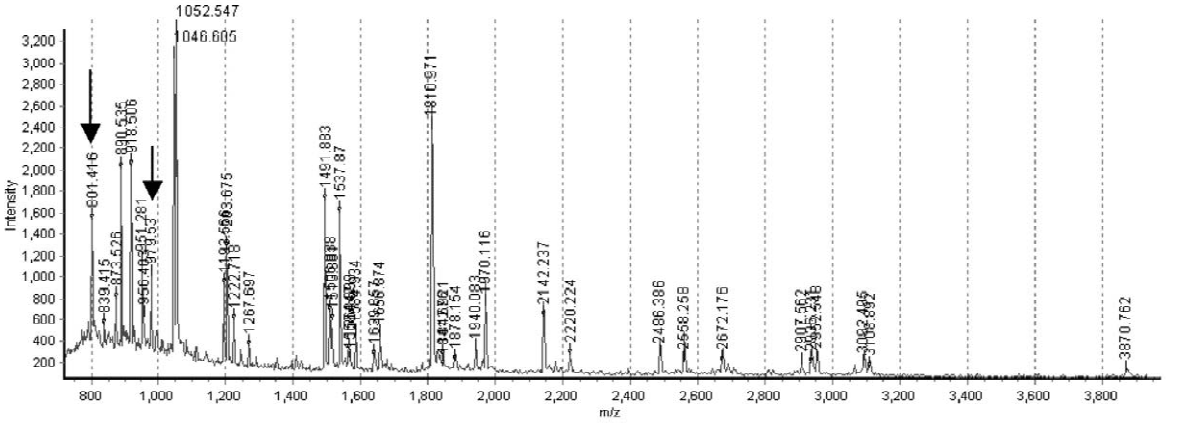


Fig. 7. MALDI-TOF peptide mass fingerprint spectrum from 250 fmol of the trypsin-digested TniA and Ettan MALDI-ToF Pro software identification results. The monoisotopic m/z values are annotated at the top of each peak. Peaks denoted by arrows (m/z 801 and 979) were chosen for sequencing by use of PSD and Ettan CAF MALDI sequencing kit.

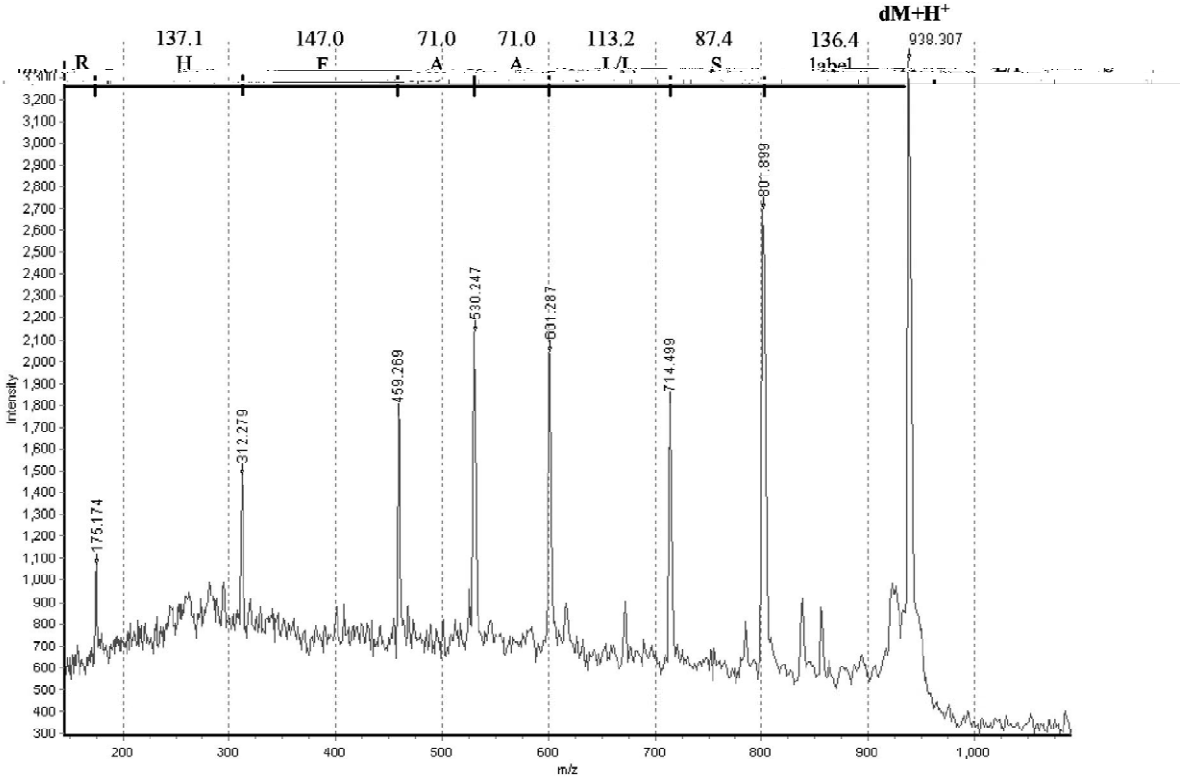
derivatization peptide 1 had a mass increase of 136 rel. mol. mass units (Arg-terminated) while peptide 2 had an increase of 42+136 rel. mol. mass units (Lys-terminated) in comparison to the underivatized masses. Due to the quadratic field reflectron of the Ettan MALDI-ToF Pro instrument, all fragments, independent of size, could be focused in a single PSD run. In contrast to a MALDI-TOF-MS instrument utilizing a linear reflectron, no time-consuming stitching procedure of several spectra covering different m/z ranges was necessary in order to acquire a complete PSD spectrum [11]. In Figs. 8 and 9 the resulting PSD spectra of the CAF derivatized peptides are shown. The complete sequences of these peptides were achieved in less than 2 min from the same sample spot that contained less than 1 pmol of material. The PepFrag [16] identification result in Fig. 8 also reveals that the average mass error for the amino acid residues of peptide 1 was less than 0.25 rel. mol. mass units with a largest deviation of 0.4 rel. mol. mass units.

4. Conclusion

The purification procedure developed consisting of anion-exchange chromatography, heparin affinity chromatography and cation-exchange chromatography resulted in a very pure (95%) TniA as judged by SDS–PAGE (Fig. 5). The fact that transposases are DNA-binding enzymes and that many of them have basic p*I* values, suggest that the presented purification protocol may serve as a general method

for the purification of recombinant non-tagged transposases and possibly other basic DNA-binding proteins.

The molecular mass of the purified TniA could be determined with high precision (within 0.1%) using MALDI-TOF-MS in the linear mode which together with results from peptide mass fingerprinting of trypsin-digested TniA resulted in an unambiguous identification of the protein covering more than 40% of the protein sequence. CAF chemistry and MALDI-PSD performed with a quadratic field reflectron can be used as a rapid and relatively accurate tool for de novo and confirmative sequencing of tryptic peptides. The results also demonstrate that MALDI-TOF-MS and the Ettan CAF Sequencing Kit are powerful means, not only for proteomics applications, but also for applications where purified protein samples need fast and accurate determinations of



gi|10955235|ref|NP_044264.1| TniA [Enterobacter aerogenes] mass = 62950.3 Da, pI = 9.6
TKQKR**SLAAFHR**EVTQV

714.50 +/- 1.00 Da: **y"6** (714.85 Da)
601.29 +/- 1.00 Da: **y"5** (601.69 Da)
530.25 +/- 1.00 Da: **y"4** (530.61 Da)
459.27 +/- 1.00 Da: **y"3** (459.53 Da)
312.28 +/- 1.00 Da: **y"2** (312.35 Da)
175.17 +/- 1.00 Da: **y"1** (175.21 Da)

Fig. 8. CAF-enhanced MALDI-PSD spectrum of the Arg-terminated peptide 1 from the tryptic digest of TniA and the resulting identification from PepFrag.

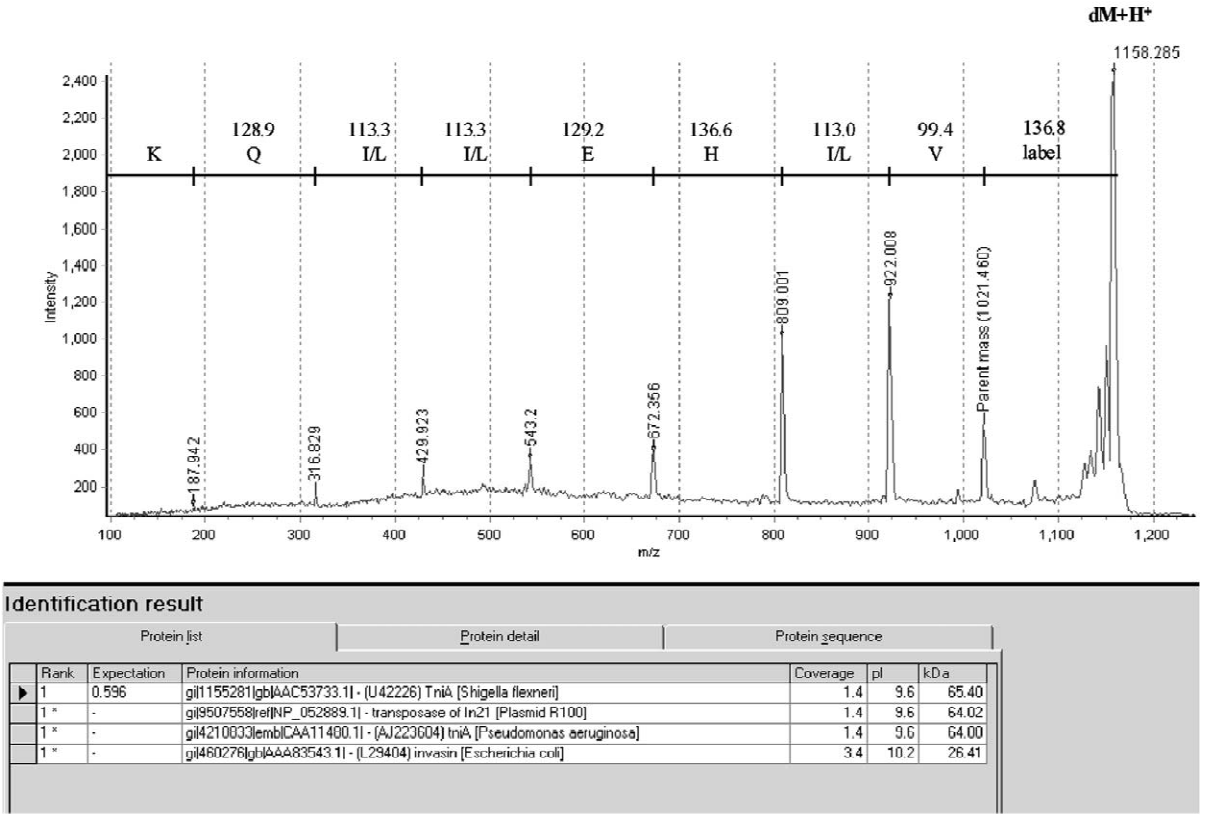


Fig. 9. CAF-enhanced spectrum of the Lys-terminated peptide 2 from the tryptic digest of TniA together with Ettan MALDI-ToF Pro software identification results.

molecular mass, identity and sequencing information from less than picomolar quantities of material.

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